BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS/ARGUMENTS

Reconsideration and continued examination of the above-identified application are respectfully requested.

The amendment to the claims is editorial in nature and/or further defines what applicants regard as their invention. The language now recites some of the language as requested by the Examiner. Full support for the amendment can be found throughout the present application, including the claims as originally filed. Accordingly, no questions of new matter should arise and entry of the amendment is respectfully requested.

Claims 1-11, 13, 15-21, and 23-29 are pending in the application, claims 12, 14, 22, and 30 have been withdrawn.

In the Office Action, the Examiner sets forth the reasons for the restriction requirement which separated the pending claims into three groups. The applicants affirm the election of Group I, which includes claims 1-11, 13, 15-21, and 23-29. The applicants believe that since claim 12 is dependent on examined claim 10, the subject matter of claim 12 should be included in the examination ongoing in the present application. Furthermore, if claim 10 is found allowable, the applicants believe at a minimum that the subject matter of claim 12 should be rejoined so that it would then be dependent on allowable subject matter. Similarly, claim 14 is dependent on claim 12 and for the same reasons should be examined at this time. Also, claim 22 is dependent on examined claim 11 and for the same reasons as claim 12, should be examined at this time. Likewise, claim 30 is dependent on claim 23, which is currently being examined. Thus, the applicants believe that with the examination of claim 23, there would be no serious burden to examine the subject matter of claim 30. At a minimum, claim 30 should be rejoined once claim 23 is found allowable. For these

reasons, the restriction requirement should be withdrawn.

At page 4, of the Office Action, the Examiner states that, while the applicants claim foreign priority under 35 U.S.C. § 119(a)-(d), the applicants have not provided an English translation of the foreign applications, and therefore the foreign priority has not been perfected. For the following reasons, this request is respectfully traversed.

The applicants respectfully contend that since the present application was a national phase entry from a PCT application, there is no need to submit a certified copy of the Japanese priority document, due to the fact that it has already been submitted by way of the PCT. Furthermore, there is no requirement that a certified English translation of a foreign priority document be submitted at all unless the Examiner questions the reliance on the benefit of that filing date, which does not appear to be the case in the present Office Action. Accordingly, the Examiner's objection to the perfection of foreign priority should be withdrawn.

At pages 4-8 of the Office Action, the Examiner rejects claims 1, 4-11, 13, 15-21, and 23-29 under 35 U.S.C. § 112, first paragraph, because the specification does not reasonably provide enablement for preparing a cell extract for cell-free protein synthesis. According to the Examiner, the material inhibiting the protein synthesis is removed by an unspecified method or through the addition of an unspecified substance, or by a method of synthesizing protein in a cell-free protein synthesis system using the cell extract alone or the cell extract and unspecified substances. The Examiner also states that the specification does not enable a person skilled in the art to make or use the invention commensurate in scope with the claims. The Examiner does indicate that the specification is enabling for: (I) the preparation of a cell-free extract for cell-free protein synthesis, wherein the substance inhibiting the protein synthesis is removed by treating the extract with a non-ionic surfactant or with the combination of a non-ionic surfactant and ultrasonication; (II) a method

of synthesizing protein in a cell-free protein synthesis system using the cell extract and other essential substances such as amino acids, energy sources, and ionic components; (III) a preparation of a cell extract wherein tritin is removed by treating a germ extract with an antibody of tritin; and (IV) a method of synthesizing protein in a cell-free protein synthesis system using the germ extract and essential substances for protein synthesis as indicated in the prior art.

The Examiner further states that claims 1, 4-11, 13, 15-21, and 23-29 encompass a preparation of a cell extract for cell-free protein synthesis, wherein the material inhibiting the protein synthesis is removed, or a method of synthesizing protein in a cell-free protein synthesis system using the cell extract. The Examiner states that the specification only discloses cursory conclusions without sufficient data supporting the findings. According to the Examiner, the specification at pages 2 and 3, states that the present invention provides for a preparation of a cell extract, excluding a system inhibiting protein synthesis for cell-free protein synthesis, a treatment of cell extracts by freeze-drying, and a method of protein synthesis using the cell-free protein system applied with molecular sieving or dialysis, at pages 2-3. The Examiner asserts that the specification is not enabling with respect to a preparation of a cell extract for cell-free protein synthesis and a method of synthesizing protein using the cell extract. The Examiner states that the present application provides no indicia and no teaching or guidance as to how the full scope of the claims are enabled.

For the following reasons, this rejection is respectfully traversed.

One skilled in the art given the guidance of the present application would be capable of removing the endosperm portion of a cell extract through a multitude of different methods, for example, water washing processing, polishing, through the use of surfactants, or through the use of surfactants and ultrasonification and the like. Listing every possible method of endosperm removal

and limiting the claims thereto is not only unnecessary, but would unduly narrow the scope of the present invention. Clearly, the scope of the claims is fully enabled by the disclosure in the present application. The present invention teaches that substantially removing the endosperm portion of a cell extract will substantially exclude the systems involved in inhibiting the cell extract's protein synthesis reactions and also control the deadenylation of ribosomes present in the remaining cell extract. The particular method of endosperm removal is clearly not critical. The present inventors have discovered that by removing or substantially excluding the endosperm portion of the cell extract, protein synthesis can be performed using any conventionally known technique. Thus, any means to remove the endosperm portion of the cell extract can be used in the present application, such as with a nonionic surfactant or other methods as detailed in the present application or other means that would be capable of excluding the endosperm portion. Thus, the present invention through great detail has provided clear enablement and predictability with respect to preparing a preparation that contains a cell extract for cell-free protein synthesis which substantially excludes the endosperm portion of the cell extract. Clearly, with the techniques and guidance set forth in the present application, one skilled in the art can clearly practice the entire scope of the present invention as claimed.

Further, the specification at page 2, line 22 through page 3, line 6 discloses examples of substances which are well known in the art, and commonly added to cell-free protein synthesis systems for the induction and continuation of protein synthesis. These substances include, for example, translation templates, energy sources, potassium and magnesium ions and the like. These substance do not generally differ whether the endosperm portion of a cell-free extract has been removed or not. The substances involved with, and the methods used for cell-free synthesis are, therefore, not unpredictable, and can be accomplished with a minimum of routine experimentation.

For these reasons, these rejections should be withdrawn.

At page 9 of the Office Action, the Examiner rejects claims 1-11, 13, 15-21, and 23-29 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that the Applicants regard as the invention.

The Examiner states that claims 1-11, 15-21, and 23-28 are indefinite because the claims recite "substantially excluding systems involving in inhibiting synthesis reaction of said own protein," and also cites an endosperm that contaminates an extract of embryo that is completely removed therefrom. The Examiner states that it is not clear whether the system involved in inhibiting protein synthesis is completely or substantially removed, and to what extent the system is excluded as to "substantially excluding systems." The Examiner states that dependent claims 2-11, 15-21, and 23-28 are included in this rejection for depending on rejected claim 1 and not correcting the deficiency of the claim from which they depend.

The Examiner also states that claims 4, 15, 16, 19, and 26 are indefinite because of the use of the term "wherein the inhibition of the own reaction of protein synthesis excluding the systems serves as controlling deadenination of ribosome." The Examiner states that the cited term renders the claim indefinite because it is not clear what the term means and that it is not clear how the system involved in the inhibition of protein synthesis also controls deadenination of ribosome, and what "deadenination" means, since neither the specification nor the prior art define the term. The Examiner states that claims 19 and 26 are included in this rejection for being dependent on the rejected independent claims and for not correcting the deficiency of the claims from which they depend. The Examiner states that the term "deadenination" also occurs in claims 5, 6, and 13

The Examiner further contends that claims 5, 6, 20, 21, 27, and 28 are indefinite because of the use of the term "a substance is added which controls deadenination of ribosome" or "a

substance controlling deadenination of ribosome." The Examiner states that the cited term renders the claim indefinite because it is not clear what the substance is.

The Examiner states that claims 7-9, and 17-20 are indefinite because the claims recites "[a] preparation" in line 1, "a substance" in line 2, and "a preparation" in line 3, and that it is not clear whether the preparation or the substance are different from each other. Dependent claims 8 and 9 are also included in this rejection.

The Examiner states that claims 10, 11, and 13 are indefinite because of the use of the phrases "a material substance," "the material substance," and "the substrate and others." The Examiner states that the cited term renders the claim indefinite because it is not clear what the material substance or the substrate and others are.

The Examiner states that claim 11 recites the limitation "the reaction vessel" in line 3, "the material substance" in line 4, and "the product" in line 5 and that there is insufficient antecedent basis for these limitations in the claims.

The Examiner states that claims 10, 11, and 23-29 are indefinite because the claims lack essential steps in the method for cell-free protein synthesis. The Examiner further argues that the omitted steps are the indispensable substances used for in vitro protein synthesis and how the protein synthesis is carried out using the cell extract and the indispensable compounds. For the following reasons this rejection is respectfully traversed.

The term "substantially" has a long precedence as an acceptably definite term. The term "excluding" is described, for instance, in the present specification at page 9 and page 11, 3-5. Also, page 11, lines 9-23 also describe the effects of the purification. Furthermore, the claims are clear and definite with respect to the fact that substantially excluding the endosperm portion of the cell extract will substantially remove the systems involved in inhibiting protein synthesis.

Further, all claims that contain the term "deadenination" have been amended to correct the spelling of the term which is "deadenylation." The applicants respectfully assert that the term "deadenylation" is described in the specification, for example, at page 11, and at page 13.

It is clear from the specification and the claims that the substance controlling the deadenylation of ribosomes can be any substance that removes the endosperm portion of the cell extract. Furthermore, at page 13, the present specification discloses the use of substances which protect ribosomes from deadenylation. One skilled in the art would be able to identify substances such as antibodies which would be capable of targeting and deactivating substances such as tritin, thionine, ribonucleic, etc. which target and deadenylate or deactivate ribosomes.

With respect to the terms "substance" and "preparation," the applicants would like to point out that a "substance" can be an individual component of a preparation, such as translation templates, energy sources, or various ions, as explained at, for example, page 2, line 22, through page 3, line 6 of the present specification. The term "preparation," on the other hand, means a finished product containing one or more substances, as defined in the present specification at, for example, page 12, line 21, through page 13, line 20 of the present specification.

The term "the material substance" is described in the present specification at, for example, page 14, line 23, through page 15, line 6. Furthermore, in claim 13, per the Examiner's suggestion, the phrase "other substances" has been substituted for the word "others." These substitutions are considered editorial in nature and do not at all change the substance of the invention as claimed.

The phrase "the reaction vessel" in line 3 of claim 11, per the Examiner's suggestion, has been replaced with the phrase "a reaction vessel." With respect to the phrase "the product" in line 5 of claim 11, per the Examiner's suggestion, the phrase has been replaced with the phrase "the synthesized product protein." These substitutions are also considered editorial in nature and do not

at all change the substance of the invention as claimed.

Contrary to the Examiner's assertion, all the relevant steps for protein synthesis are found in the claims. With respect to this rejection, the Examiner asserts that claims 10, 11, and 23-29 are indefinite because the claims lack essential steps in the method for cell-free protein synthesis. However, this rejection is not completely understood under 35 U.S.C. § 112. 35 U.S.C. § 112, with respect to definiteness, only requires that the claims be clear and definite when one skilled in the art reads the claims. Clearly, these claims are clear and definite to one skilled in the art. The Examiner does not appear to assert that these claims cannot be understood but only seems to assert, with respect to the part of this rejection, that the claims lack essential steps. However, there is no requirement under 35 U.S.C. § 112 that would address the Examiner's assertions with respect to the lack of essential steps. Under 35 U.S.C. § 112, the claims are required to be enabling and to be definite. Clearly, the claims satisfy all provisions of 35 U.S.C. § 112. In addition, the specification provides clear guidance to enable the complete scope of these claims. Accordingly, this rejection should be withdrawn.

Furthermore, all the substances useful for cell free protein synthesis are found in the specification at, for example, page 12, line 21, through page 13, line 20. It is well known to one skilled in the art that the protein synthesis can be carried out using methods and apparatuses as disclosed in the application and discussed above. Therefore, the Examiner's rejection under 35 U.S.C. § 112 should be withdrawn.

At page 11, of the Office Action, the Examiner rejects claims 1, 4, 5, 23, 26, and 27 under 35 U.S.C. § 102(b) as being anticipated by Japanese Publication No. 07-203984, published on August 8, 1995, to Endou Yaeta et al. The Examiner states that the rejection is based on an electronic translation of the patent publication from the Japanese Patent Office and that an English

translation of the publication will be forwarded to us when it is available. The Examiner states the Endou teaches a ribosome inactivation protein, named Torichin (tritin), found in wheat germ that can inactivate ribosomes by removing an adenine from 28S rRNA, at paragraph 0019 of the reference, and that a wheat germ extract is prepared by removing Torichin activity by column and adding Torichin antibody, at paragraph 0029, claims 1, 4, and 5 of the reference. The Examiner states that the efficiency of protein synthesis in a wheat germ cell-free protein synthesis system is increased by using Torichin antibody and removing neutralized Torichin, as found at paragraphs 0031-0038, drawings 2 and 3, examples 2 and 3, and claims 23, 26, and 27 of the reference. For the following reasons, this rejection is respectfully traversed.

With respect to this rejection, the applicants respectfully point out that a complete formal English translation of the publication has not been provided to the applicants. Accordingly, the applicants are responding to this rejection in view of the electronic translation of the Japanese Patent Office, which may not be completely accurate. In view of these circumstances and in view of the fact that the applicants have not received a true English translation of the publication, the applicants believe that any subsequent Office Action, should it be necessary, clearly needs to be non-final due to these circumstances.

Endou only discloses a wheat germ extract prepared by adding tritin antibodies to an affinity column and removing tritin therewith. Evasion of contaminants other than tritin derived from the endosperm and the extract cannot be accomplished by the method described in Endou. These other contaminants have a substantial affect on protein synthesis. Furthermore, the process described for making a cellular extract in Endou is complicated, involves high cost, and does not preclude endosperm contamination. The following table shows differences between the present invention and Endou and the effect of endosperm contaminants other than tritin.

	Endou Fig.2 of specification (JP-07203984) (Attachment 2)	This invention Proc.Natl.Acad.Sci.USA vol.97 p559-564 2000, Fig 2 (The author is same as this inventor: Attachment 3)
Method of preparing cell extract	Endosperm is contaminated in cell extract.	Endosperm is not substantially contaminated in cell extract by introducing the process washing with surfactant.
Activity of protein synthesis	9500 dpm/5μl(4hr) ① 7800 dpm/5μl(4hr) ②	2400 dpm/5μl(4hr) ④
Conventional (as control)	4000 dpm/5μl(4hr) ③	700 dpm/5μl(3hr) ⑤

The chart above summarizes the result of two separate experiments, included as attachments 2 and 3. The first experiment (attachment 2) shows the amount of protein synthesis produced using the traditional cell-free methods (3), the amount of protein produced using the Endou disclosed tritin antibody method (2), and the amount of protein produced through the Endou disclosed tritin antibody and column distillation method (1). Protein production is measured using the incorporation of C^{14} Leucine. The chart shows that removing tritin by antibody alone increases protein production versus traditional methods by about 1.95 times, and removing tritin by antibody and column distillation increases protein production versus traditional methods by about 2.4 times.

The chart further summarizes the results of a second set of experiments (attachment 3) which measures (using C^{14} Leucine) the amount of protein prepared using the present invention (4) and the conventional method (5). The amount of protein prepared by the present invention is about

3.4 times the amount produced by the traditional method (the amount of protein produced by the traditional method is the same after 4 hours as it is after three hours as shown.)

The amount of C^{14} Leucine incorporated into the first and second experiments is not comparable as the experiments involved different concentrations of the isotope. For comparison purposes Endou and the present invention each must be compared against the results obtained from the conventional method practiced under the same conditions. Protein synthesis increased about 2.4 fold between the conventional method and Endou. Protein synthesis increased about 3.4 between the conventional method and the present invention. The increase of 2.4 to 3.4 shows the unexpected and superior effect of the present invention as compared to Endou.

Attachments 1-4 have been included to further demonstrate the advantages in the present invention when compared to Endou and conventional methods. The applicants are willing to submit this data in the form of a § 1.132 Declaration if the Examiner so requires.

Therefore, the present invention provides for the removal of protein synthesis degrading contaminants besides tritin by treating the extract with the methods described in the present application. In the present invention, evasion of the contaminants besides tritin derived from endosperm in an extract of an embryo can be performed as set forth in the claims. For instance, the extract can be treated with a nonionic surfactant to achieve the goals of the present application. This novel and useful preparation and the method to accomplish it are clearly not taught or suggested in Endou. This allows one skilled in the art to produce a high efficiency protein synthesis reaction having a longer reaction time. In addition, the applicants wish to inform the Examiner that there has been commercial success of this invention with respect to a kit identified by the tradename of Proteios™. *See* Attachment 1. Accordingly, the Examiner's rejection under 35 U.S.C. § 102 over Endou should be withdrawn.

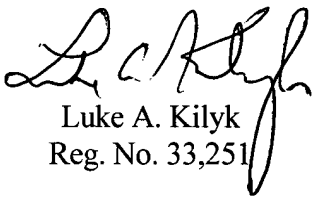
Should the Examiner have any questions, the Examiner is encouraged to contact the undersigned by telephone.

CONCLUSION

In view of the foregoing remarks, the applicant respectfully requests the reconsideration of this application and the timely allowance of the pending claims.

If there are any other fees due in connection with the filing of this response, please charge the fees to Deposit Account No. 50-0925. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such extension is requested and should also be charged to said Deposit Account.

Respectfully submitted,



Luke A. Kilyk
Reg. No. 33,251

Atty. Docket No. 3190-012
KILYK & BOWERSOX, P.L.L.C.
53 A East Lee Street
Warrenton, VA 20186
Tel.: (540) 428-1701
Fax: (540) 428-1720
Enclosures: Attachments 1-4

Attachment 1-1

PROTEIOS™ PROTOCOL

January 8, 2001

I. *in vitro* transcription

DW	36-(Plasmid soln.) μl
10×T7 buffer*	5
25mM NTPs*	5
RNasin(40U/μl)*	1
Plasmid soln.	5(μg)
T7 RNA polymerase(50U/μl)*	3

*Not attached

・Incubate the reaction mixture at 37℃ for >3h.

(One hundred microliter reaction gives 120-160μg RNA)

II. Purification of the transcripts using MicroSpin™ G-25 Columns (Amersham Pharmacia Biotech Inc.)

1. Resuspend the resin in the columns by vortexing gently.
2. Loosen the cap and snap off the bottom closure.
3. Place the column in a 1.5ml screw-cap microcentrifuge tube for support.
4. Pre-spin the column for 1 minute at 735xg (3,000r.p.m.).
5. Place the column in a new 1.5ml tube and apply 200μl of Buffer mix*.
(*For batch synthesis, 20mM HEPES-KOH (pH7.6) should be used)
6. Spin the column for 2 minutes at 735xg(3,000r.p.m).
7. Repeat 5-6 for 2 times.
8. Place the column in a new 1.5ml tube and apply 100μl of transcript.
9. Spin the column for 2 minutes at 735xg(3,000r.p.m.).
10. Discard the column.
11. Measure the OD260nm of a 1:100 dilution (in water) of the solution, and calculate the concentration of the RNA(1 OD260nm= 40μg RNA). <Control: 1:100 dilution of Buffer mix (for diffusion method) or HEPES-KOH(pH 7.6) (for batch method))>.
12. Store on ice.

Attachment 1-2

(Buffer mix)

Buffer #1	1.07(ml)
Buffer #2	1.25
DW	7.68
	10 (ml)

Ⅲ. *In vitro* translation

<Batch method>

1. Prepare the RI mix

(1sample)

Buffer #2	3 μl
Creatine kinase (10mg/ml)	1
([^{14}C] Leucine)*	1

*L-[U-^{14}C] Leucine(1.85MBq, 50μCi/ml) Amersham pharmacia biotech. (Code No.,CFB183)

2. Prepare the Reaction Mix

(1sample分)

DW	12.8 − (mRNA soln.) μl
Buffer #1	1.7
mRNA solution / DW	6(μg)
RNase inhibitor (40U/μl)	0.5
Wheat germ extract	5.0
RI mix	5.0

3. Inoubate at 23-26℃ for 1-3hours.

4. Detect the radioactivity by spot test.

Attachment 1-3

<Diffusion method>

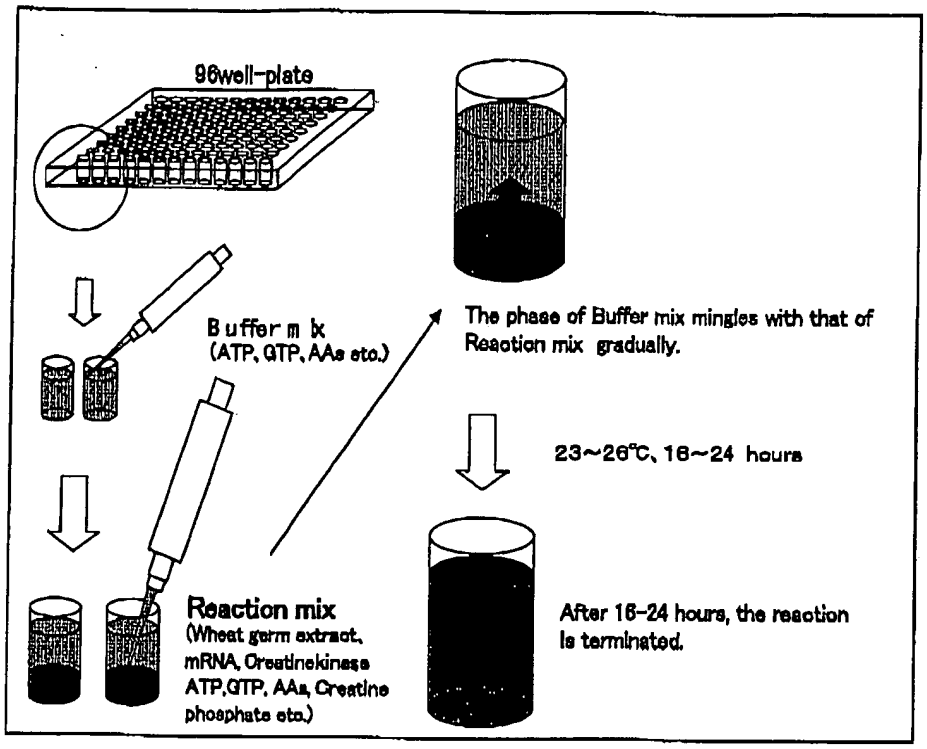


Fig 1 Principle and flow-chart of the "Diffusion method".

1. Preparation of Reaction mix (50 μl).

DW	1.8 μl
Buffer #2	2.0
Creatine kinase(10mg/ml)	1.7
RNase Inhibitor(40U/μl)*	1.0
Wheat germ extract	10.0
RNA solution(0.3-0.4 μg/μl) / Buffer mix	33.5

*Not attached

2. Fill a well of 96-well plate with 250 μl of Buffer mix.

3. Apply the Reaction mix (50 μl) to the bottom of the well carefully.. (→Fig 2)

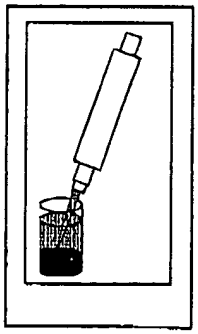


Fig 2

4. Incubate the plate at 23-26°C for 16-24 hours.

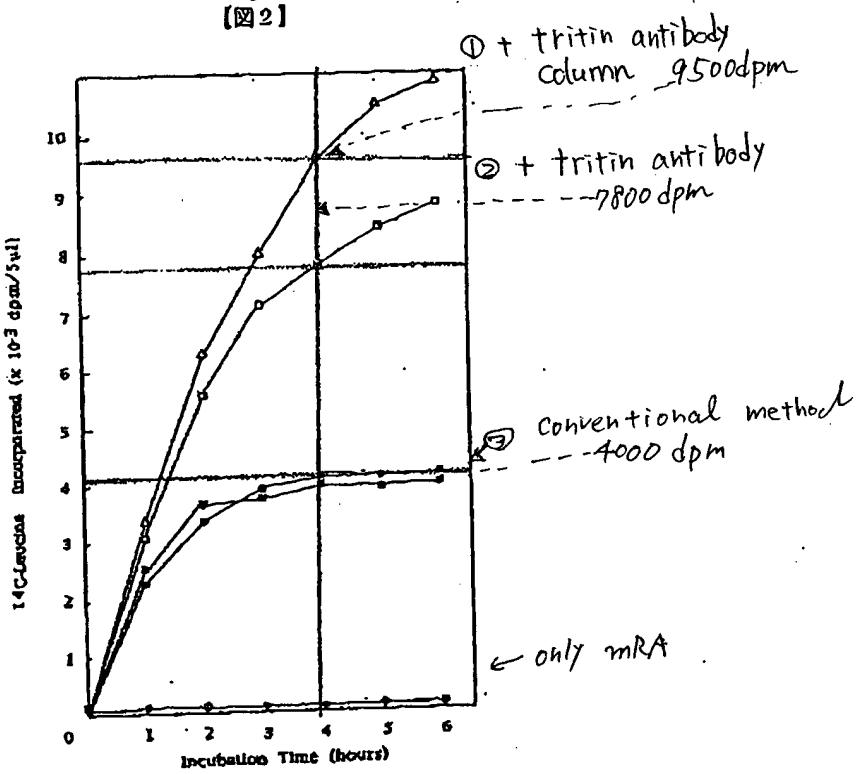
5. Recover 300 μl of the reaction mixture.

Attachment 2

添付資料①

特許3255784

Fig2
【図2】



フロントページの続き

(56)参考文献　特開 平5−236986 (JP, A)
Biochem. Biophys. Res. Commun. (1983) Vol. 114, No. 1, p. 190−196

(58)調査した分野(Int.Cl.[7], DB名)
C12P 21/00 - 21/02
C12N 15/09
BIOSIS (DIALOG)
WPI (DIALOG)

The measuring method of ^{14}C-Leucine incorported amount (Fig2) is the described in "Erickson, A.H., et al Methods in Enzymology, 96, 38-50 1983" (Attachment 4 : p41 ~ 42)

as determined as follows: Aliquots were
ples containing 1 μl of reaction mixture were
% SDS polyacrylamide gels (8% gels for TMV
% native polyacrylamide gels (for GFP), then
th Coomassie brilliant blue. The product amount
d by densitometric scanning of the bands and
to standards. The standard samples were prepared
g a reaction mixture without mRNA with known
s of standard proteins (DHFR, GFP, or luciferase)
loading onto the gel. Because pure, authentic, 126-kDa
protein is not available, the amount of this protein was
mated with less accuracy by calculating its relative amount
ompared with molecular markers included as internal standards
by using average 105- and 160-kDa band intensities. The amount
of DHFR was confirmed by determining the amount of meth-

report of solvent flotation for the enrichment of viable
embryos from wheat seeds by Johnston and Stern (2
method has commonly been used for the preparation of
embryos. We first addressed the possibility of a tritin co
nation originating from endosperm as the reason for the
bility of wheat germ cell-free systems. If wheat germs are i
from dry wheat seeds by conventional procedures (27),
scopic examination reveals that the sample contains emb
well as some white material and a number of white and br
granules (Fig. 1A). Analysis of ribosomal RNAs from a p
synthesis reaction prepared from such a sample showe
depurination of ribosomes occurs, contradicting earlier r
(20–22) (Fig. 1B). After 4 h of incubation, 24% of the rib
population had been depurinated, as judged by the a
dependent formation of a specific RNA fragment (Fi

Attachment 3. 添付資料②

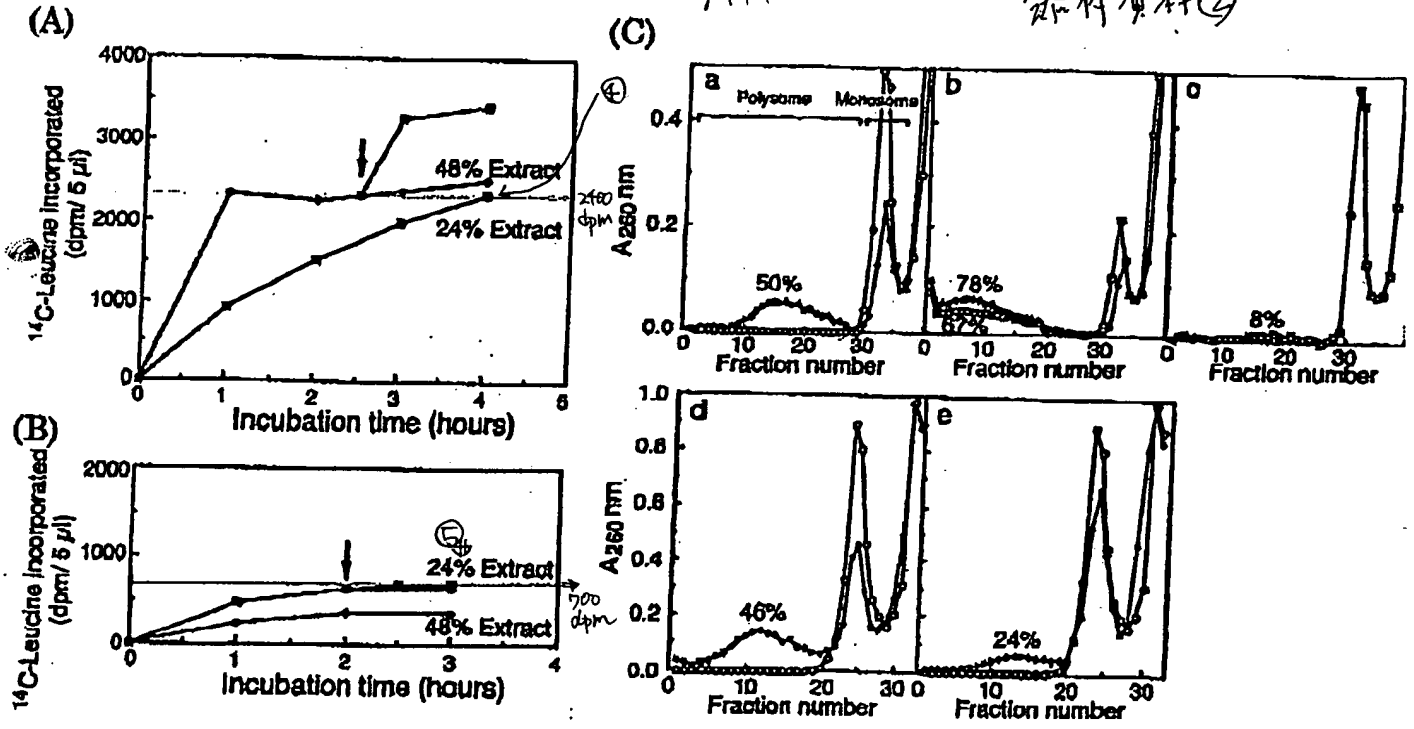


Fig. 2. Protein synthesis with an extract prepared from washed embryos. The batch system contains either 12 μl (24%) or 24 μl (48%) of extracts from wa
(A) c nwashed (B) wheat embryos. Protein synthesis was measured as hot trichloroacetic acid insoluble radioactivity. Arrows show addition of substrat
shov. the polysome profiles of 15 μl of reaction mixture aliquots loaded onto a linear 10% to 45% sucrose gradient in 25 mM Tris·HCl (pH 7.6), 100 mM
and 5 mM $MgCl_2$. After centrifugation, fractions were collected from the bottom of the tubes and were measured at 260 nm as described (24). Incubation t
were 0 h (open circles in a), 1 h (closed circles in a), and 2 h (b) in the absence (open circles in b) or presence (closed circles in b) of 0.4 μM cycloheximide.
The translation system prepared from unwashed embryos was incubated for 2 h. In d and e, aliquots from the dialysis system were withdrawn after 48 and
and were incubated in the presence of 0.4 μM cycloheximide for another 60 min at 26°C (closed circles). Similar analyses of the samples were carried out ir
absence of mRNA (d and e, open circles) as negative controls.

④ the method of this invention

⑤ conventional method

(From the curve of graph,
it may be 700 dpm/5μl at the time 4 hours)

Reference p560 line5~6.

P.561

Attachment 3

A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: Plants apparently contain a suicide system directed at ribosomes

Kairat Madin, Tatsuya Sawasaki, Tomio Ogasawara, and Yaeta Endo*

Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790-8577, Japan

Edited by Harry F. Noller, University of California, Santa Cruz, CA, and approved November 15, 1999 (received for review August 17, 1999)

Current cell-free protein synthesis systems can synthesize proteins with high speed and accuracy, but produce only a low yield because of their instability over time. Here we describe the preparation of a highly efficient but also robust cell-free system from wheat embryos. We first investigated the source of the instability of existing systems in light of endogenous ribosome-inactivating proteins and found that ribosome inactivation by tritin occurs already during extract preparation and continues during incubation for protein synthesis. Therefore, we prepared our system from extensively washed embryos that are devoid of contamination by endosperm, the source of tritin and possibly other inhibitors. In a batch system, we observed continuous translation for 4 h, and sucrose density gradient analysis showed formation of large polysomes, indicating high protein synthesis activity. When the reaction was performed in a dialysis bag, enabling the continuous supply of substrates together with the continuous removal of small byproducts, translation proceeded for >60 h, yielding 1–4 mg of enzymatically active proteins, and 0.6 mg of a 126-kDa tobacco mosaic virus protein, per milliliter of reaction volume. Our results demonstrate that plants contain endogenous inhibitors of translation and that after their elimination the translational apparatus is very stable. This contrasts with the common belief that cell-free translation systems are inherently unstable, even fragile. Our method is useful for the preparation of large amounts of active protein as well as for the study of protein synthesis itself.

The development of a system capable of synthesizing any desired protein on a preparative scale is one of the most important endeavors in biotechnology today. Three strategies are currently being used: chemical synthesis, *in vivo* expression, and cell-free protein synthesis. The first two methods have severe limitations: chemical synthesis is not feasible for the synthesis of long peptides because of low yield, and *in vivo* expression can produce only those proteins that do not affect the physiology of the host cell (1–3). Cell-free translation systems, in contrast, can synthesize proteins with high speed and accuracy, approaching *in vivo* rates (4–5), and they can express proteins that would interfere with cell physiology. However, they are relatively inefficient because of their instability (6).

Because cell free systems nonetheless have great potential for large scale protein synthesis, many efforts have been made to increase their efficiency. Spirin *et al.* (7) proposed a continuous flow cell-free translation system, in which a solution containing amino acids and energy sources is supplied to the reaction chamber through a filtration membrane. This design is significantly more efficient than conventional batch systems: The reaction works for tens of hours and produces hundreds of micrograms per milliliter of reaction volume (7–9). Recently, several modified versions of the Spirin system have been reported (10–13). Kigawa *et al.* showed that, by using a dialysis membrane to facilitate the continuous supply of substrates and removal of byproducts, an *Escherichia coli*-coupled transcription–translation system yields as much as 6 mg of protein per milliliter of reaction volume (12). This high productivity can, however, only be expected with fairly small proteins such as Ras protein (21 kDa) or chloramphenicol acetyltransferase (26 kDa). The problem with larger proteins is that with the increasing molecular weight of the mRNAs their degradation by endogenous *E. coli* ribonuclease(s) also increases. Kawarasaki *et al.* showed that in a wheat germ cell-free system translational efficiency increases after neutralization of endogenous ribonucleases and phosphatases with copper ions and antiphosphatase antibodies (13). For their improvements, these groups focused on modifying the reaction chamber and/or optimizing the reaction conditions while using conventional extracts. We used a different approach, instead focusing on clarifying the nature of the instability of the extracts.

We concentrated on wheat germ cell-free systems because they have numerous advantages such as low cost, easy availability in large amounts, low endogenous incorporation, and the capacity to synthesize high-molecular-weight proteins. Moreover they are eukaryotic systems and hence more suitable for the expression of eukaryotic proteins. After we discovered that the mechanism of action of the ricin toxin is ribosome inactivation (14–16), many other ribosome-inactivating proteins (RIPs) with identical mechanism of action have been found in higher plants (17). Most commonly these toxins are single-chain proteins, and they inhibit protein synthesis by removing a single adenine residue in a universally conserved stem-loop structure of 28S ribosomal RNA (14–17). Although the biological function of the RIPs is not known, it is generally believed that they are important for cell defense (17). The most widely studied example is an antiviral effect during infection by several plant viruses (18). As originally proposed by Ready *et al.* (19), the explanation for the antiviral activity of RIPs is that, when a cell wall is damaged, the RIP is released into the cytosol, where it inactivates ribosomes, thereby preventing virus replication. Tritin, found in wheat seeds and thought to be localized mainly in the endosperm, is such a single-chain RIP (20). Initially, it was reported that wheat embryonic ribosomes are resistant to this protein (20–22), which would render any contamination with tritin inconsequential.

To improve protein synthesis in wheat germ cell-free systems, we started with the hypothesis that the embryonic ribosomes are in fact susceptible to tritin. In this case, contamination of wheat germ preparations with tritin-containing endosperm fragments would be fatal. Accordingly, we prepared our cell-free system

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: RIP, ribosome-inactivating protein; TMV, tobacco mosaic virus; GFP, green fluorescent protein; DHFR, dihydrofolate reductase.

*To whom reprint requests should be addressed. E-mail: yendo@en3.ehime-u.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

from extensively washed embryos and indeed found that the system became far more active.

In addition to the benefit of a better protein synthesis system, these results shed new light on the translational apparatus itself: Although it is usually seen as a rather fragile apparatus, it appears instead to be very stable: so stable, in fact, that plants seem to have developed a suicide mechanism (the RIPs) directed against the translational apparatus, further emphasizing its crucial role in cell physiology. We believe that the strategy we followed to improve the wheat cell-free system—elimination of endogenous translational inhibitors—is equally applicable for other systems.

Materials and Methods

General. The following procedures were either described or cited previously (9, 14–15, 23–24): determination of RNA *N*-glycosidase activity, analysis of cell-free protein synthesis, sucrose density gradient analysis of polysomes, determination of proteins, the sources of m7GpppG, ribonucleotide triphosphates, SP6 RNA polymerase, T7 RNA polymerase, human placental ribonuclease inhibitor (133 units/ml), L-[U-^{14}C]leucine, MTX immobilized on agarose, creatine kinase, spermidine, and the 20 amino acids. Dialysis membrane (molecular weight cutoff 12,000–14,000, regenerated cellulose, Viskase Sales, Chicago), the nonionic detergent Nonidet P-40, and proteinase inhibitor E64 were purchased from Nakarai Tesque (Kyoto). The luciferase assay kit (PiccaGene) was from Wako Pure Chemical (Osaka). Low and high molecular weight marker kits (Rainbow marker) were from Amersham Pharmacia. Recombinant forms of luciferase and green fluorescent protein (GFP) (S65T) that were used as standards were purchased from Promega and CLONTECH, respectively. Plasmid pCaMV35S-sGFP(S65T)-NOS3′(25) carrying the GFP gene was kindly provided by Y. Niwa (School of Food and Nutritional Sciences, University of Shizuoka, Japan), and plasmid pSP-Luc$^+$ carrying luciferase was obtained from Promega. Plasmid pTLW3 (26), covering the tobacco mosaic virus (TMV) genome, was a generous gift from Y. Watanabe (University of Tokyo).

Purification of Wheat Embryos and Extract Preparation. Wheat seeds were ground in a mill (Roter Speed Mill model pulverisette 14, Fritsh, Germany), then were sieved through a 710- to 850-mm mesh. Embryos were selected with the solvent flotation method of Erickson and Blobel (27) by using a solvent containing cyclohexane and carbon tetrachloride (240:600, vol/vol). Damaged embryos and contaminants were discarded, and intact embryos were dried overnight in a fume hood. To remove contaminating endosperm, the embryos were washed three times with 10 vol of water under vigorous stirring, and then were sonicated for 3 min in a 0.5% solution of Nonidet P-40 by using a Bronson model 2210 sonicator (Yamato, Japan). Finally, the embryos were washed once more in the sonicator with sterile water.

Preparation of the Cell-Free Extract. The method used is a slight modification of the procedure described by Erickson and Blobel (27). Washed embryos were ground to a fine powder in liquid nitrogen. Five grams of the powder were added to 5 ml of 2 × buffer A (40 mM Hepes, pH 7.6/100 mM potassium acetate/5 mM magnesium acetate/2 mM calcium chloride/4 mM DTT/0.3 mM of each of the 20 amino acids). The mixture was briefly vortexed and then was centrifuged at 30,000 × *g* for 30 min. The resulting supernatant was subjected to gel-filtration on a G-25 (fine) column, equilibrated with two volumes of buffer A. The void volume was collected and centrifuged at 30,000 × *g* for 10 min. The final supernatant was adjusted to 200 A260/ml with buffer A, was divided into small aliquots, and was stored in liquid nitrogen until use.

Cell-Free Translation. In the batch system, 50 μl of reaction mixture contained 12.5 μl of extract (thus 24%); final concentrations of the various ingredients are 24 mM Hepes/KOH (pH 7.8), 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.45 mg/ml creatine kinase, 2 mM DTT, 0.4 mM spermidine, 0.3 mM of each of the 20 amino acids including [^{14}C]leucine (2 μCi/ml), 2.5 mM magnesium acetate, 100 mM potassium acetate, 50 μg/ml of deacylated tRNA prepared from wheat embryos, 0.05% Nonidet P-40, 1 μM E-64 as proteinase inhibitor, 0.005% NaN_3, and 7.2 μg (0.02 nmol) of dihydrofolate reductase (DHFR) mRNA. The extract was not treated with micrococcal nuclease because we did not observe any positive effect of this treatment. Incubation was done at 26°C.

For the dialysis system, 500 μl of reaction mixture contained 300 μl of the extract and the same ingredients as described above. The dialysis bag was immersed in 5 ml of a solution containing all described ingredients except for creatine kinase. The reaction was done at 23°C, and, every 24 h, 0.05 nmol of DHFR mRNA (or equivalent moles of the other mRNAs) and 50 μg of creatine kinase were supplemented. The dialysis solution was also replaced every 24 h. To confirm the longevity of the system, [^{14}C]leucine (the same concentration as above) was added into both reaction mixture and dialysis buffer at 52 h, then was incubated until 72 h (Fig. 4*C*). The autoradiogram of the gel was obtained by using a BAS-2000 phosphoimager (Fuji).

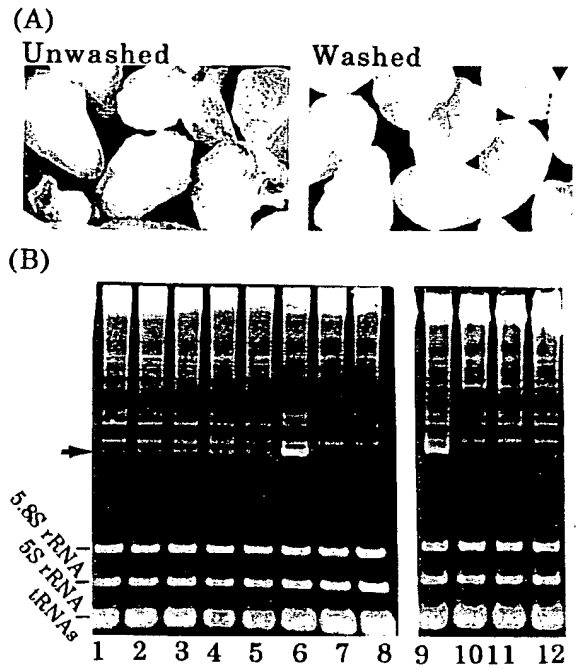


Fig. 1. Removal of tritin from embryos. Extracts were prepared from unwashed or washed embryos (*A*), and the depurination assay was performed (*B*). Translation mixtures prepared with the extract from unwashed embryos were incubated for 0, 1, 2, 3, and 4 h (*B*, lanes 1–5 respectively); mixtures with washed embryos were incubated for 0, 2, and 4 h (lanes 10–12, respectively). Isolated RNA was treated with acid/aniline, then was separated on 4.5% polyacrylamide gels. Additionally, RNA was directly extracted from embryos with guanidine isothiocyanate-phenol and was analyzed as above before (*B*, lane7) and after (*B*, lane 8) treatment with acid/aniline. For the fragment marker (*B*, lanes 6 and 9), incubation was carried out in the presence of gypsophilin (40), a highly active RIP from *Gypsophila elegance*; the arrow indicates the aniline-induced fragment.

Preparation of mRNA. Capped mRNA encoding DHFR was synthesized by *in vitro* transcription of linearized plasmid pSP65 carrying the gene under SP6 RNA polymerase promoter control (9). The transcript is 1,079 nucleotides long and consists of the sequence m^7GpppGAAUACACGGAAUUCGAGCUCG-CCCGGGAAAUCUCA*AUG* (the italicized sequence is the initiation codon) at its 5′ end, a 477-nt coding sequence, and a 3′ noncoding region of 565 nucleotides with a poly(A) tail of 100 adenosines (9). Coding sequences for GFP (717 nucleotides) (25) and luciferase (1,650 nucleotides) were cloned into the above plasmid in such a way that the 5′- and 3′-untranslated regions of DHFR were preserved. Capped TMV RNA (6,388 nucleotides) was transcribed from linearized plasmid pTLW3 carrying the genome under T7 RNA polymerase promoter control (26).

Analysis of Products and Their Enzymatic Activities. The amount of protein synthesized was determined as follows: Aliquots were withdrawn, and samples containing 1 μl of reaction mixture were separated on 12.5% SDS polyacrylamide gels (8% gels for TMV protein) or 12.5% native polyacrylamide gels (for GFP), then were stained with Coomassie brilliant blue. The product amount was estimated by densitometric scanning of the bands and comparison to standards. The standard samples were prepared by mixing a reaction mixture without mRNA with known amounts of standard proteins (DHFR, GFP, or luciferase) before loading onto the gel. Because pure, authentic, 126-kDa TMV protein is not available, the amount of this protein was estimated with less accuracy by calculating its relative amount compared with molecular markers included as internal standards by using average 105- and 160-kDa band intensities. The amount of DHFR was confirmed by determining the amount of methotrexate-agarose column purified protein, and its activity was measured colorimetrically as described (9). Luciferase activity was determined by using a commercial kit and a liquid scintillation counter as described (28). The specific activities of recombinant luciferase and the synthesized protein were 3.4×10^5 and 5.1×10^6 cpm/pg, respectively. Semiquantitative measurement (28) of GFP activity on the native gel was carried out by using a UV-illuminator (Dark Reader, Clare Chemical Research, Denver) with a wavelength of 400–500 nm. Subsequent scanning of photographs of the UV images and comparison of the intensities of the bands to those of the recombinant protein revealed that the translation product had more activity than the standard by a factor of 1.4.

Results and Discussion

Removal of Contaminants such as Tritin from Wheat Embryos Leads to a More Active Cell-Free Protein Synthesis System. Since the first report of solvent flotation for the enrichment of viable, intact embryos from wheat seeds by Johnston and Stern (29), this method has commonly been used for the preparation of wheat embryos. We first addressed the possibility of a tritin contamination originating from endosperm as the reason for the instability of wheat germ cell-free systems. If wheat germs are isolated from dry wheat seeds by conventional procedures (27), microscopic examination reveals that the sample contains embryos as well as some white material and a number of white and brownish granules (Fig. 1*A*). Analysis of ribosomal RNAs from a protein synthesis reaction prepared from such a sample showed that depurination of ribosomes occurs, contradicting earlier reports (20–22) (Fig. 1*B*). After 4 h of incubation, 24% of the ribosome population had been depurinated, as judged by the aniline-dependent formation of a specific RNA fragment (Fig. 1*B*,

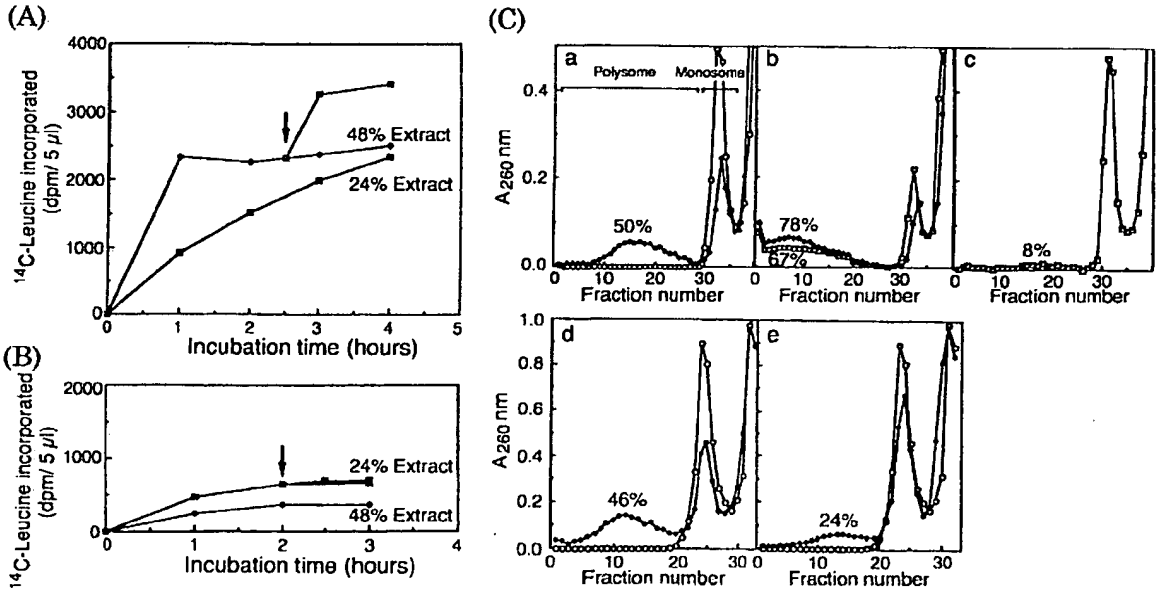


Fig. 2. Protein synthesis with an extract prepared from washed embryos. The batch system contains either 12 μl (24%) or 24 μl (48%) of extracts from washed (*A*) or unwashed (*B*) wheat embryos. Protein synthesis was measured as hot trichloroacetic acid insoluble radioactivity. Arrows show addition of substrates. C shows the polysome profiles of 15 μl of reaction mixture aliquots loaded onto a linear 10% to 45% sucrose gradient in 25 mM Tris·HCl (pH 7.6), 100 mM KCl, and 5 mM $MgCl_2$. After centrifugation, fractions were collected from the bottom of the tubes and were measured at 260 nm as described (24). Incubation times were 0 h (open circles in *a*), 1 h (closed circles in *a*), and 2 h (*b*) in the absence (open circles in b) or presence (closed circles in *b*) of 0.4 μM cycloheximide. In c, the translation system prepared from unwashed embryos was incubated for 2 h. In *d* and e, aliquots from the dialysis system were withdrawn after 48 and 60 h and were incubated in the presence of 0.4 μM cycloheximide for another 60 min at 26°C (closed circles). Similar analyses of the samples were carried out in the absence of mRNA (*d* and *e*, open circles) as negative controls.

arrow). Furthermore, even at the start of the incubation, 7% of the population had already been depurinated. The site of depurination was confirmed by direct sequencing of the fragment to be in the universally conserved sarcin/ricin domain of 28S rRNA (data not shown). When RNA was extracted directly from embryos by guanidine isothiocyanate-phenol, little formation of the aniline-induced fragment was observed (Fig. 1*B*, lanes 7 and 8). Thus, depurination must have occurred during the extract preparation and then continued during the protein synthesis reaction.

The observed extent of depurination constitutes a considerable damage to protein synthesis because inactivation of any one ribosome among the actively translating ribosomes on an mRNA results in blockage of the respective polyribosome and cessation of translation (16). Attempts were made to neutralize the depurinating enzyme with synthetic RNA aptamers that tightly bind to the RIP (30), but these attempts failed. Instead, careful selection and subsequent extensive washing of the embryos yielded better results. These embryos had few contaminants (Fig. 1*A Right*), and when the depurination assay was performed, no aniline-induced cleavage was detectable (Fig. 1*B*, lanes 10–12), indicating minimal, if any, depurination during preparation as well as incubation.

As shown in Fig. 2, the cell-free system prepared from washed embryos has much higher translational activity than the conventional system (compare Fig. 2 *A* and *B*). When programmed with mRNA coding for DHFR, it has almost linear kinetics in DHFR synthesis over 4 h in a system containing 24% extract, as opposed to the regular system, which ceased to function after 1.5 h. When the content of washed extract in the reaction volume was increased to 48%, amino acid incorporation occurred initially at a rate twice that with 24% extract, but then stopped after 1 h. However, this halting was caused by a shortage of substrates rather than an irreversible inactivation of ribosomes or factors necessary for translation: Addition of amino acids, ATP, and GTP after cessation of the reaction (Fig. 2 *A* and *B*, arrows) restarted translation with kinetics similar to the initial rate. In contrast, if conventional extract was added to 48%, protein synthesis actually decreased compared with the 24% extract reaction. Furthermore, the halting of protein synthesis in the

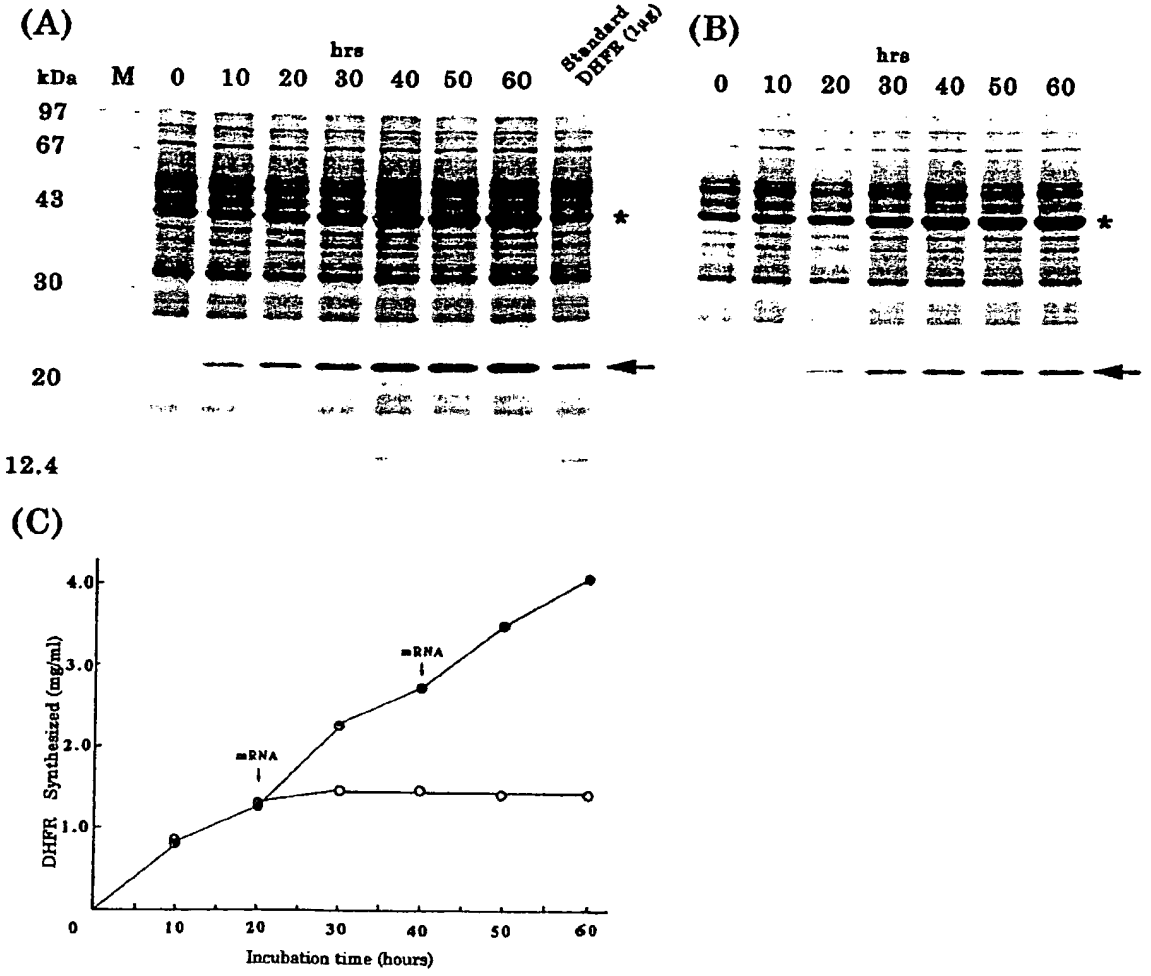


Fig. 3. Protein synthesis in the dialysis system. (*A* and *B*) Coomassie blue-stained SDS polyacrylamide gels showing DHFR synthesis with (*A*) or without (*B*) addition of new mRNA. Arrows and asterisks mark DHFR and creatine kinase, respectively. The standard sample was prepared by mixing a reaction mixture without mRNA with known amounts of DHFR before loading onto the gel. (*C*) Amounts of DHFR synthesized as determined from densitometric scans of the gels in *A* (closed circles) and *B* (open circles).

reaction with 24% extract could not be reversed by the addition of more substrate, indicating an irreversible damage by contaminants from endosperm (Fig. 2*B*).

High protein synthesis activity of the system with washed embryos can also be demonstrated by sucrose density gradient analysis (Fig. 2*C*). Significant formation of polysomes was observed after 1 h of incubation, and at 2 h a shift to heavier polysomes with a concomitant decrease of 80S monosomes was seen (Fig. 2*C a* and *b*). In the presence of low concentrations of cycloheximide polysome formation is a measure of translational initiation (31). A concentration of cycloheximide of 0.4 μM reduced the incorporation of [^{14}C]leucine to 21% of the control (data not shown) and resulted in an accumulation of large polysomes, with 78% of ribosomes in polysomes (open circle in Fig. 2*C b*). A similar analysis of cell-free reactions prepared with regular extracts (27), but done in the absence of cycloheximide, did not show significant polysome formation (Fig. 2*C c*). The high efficiency of our system, therefore, can be attributed to at least two factors: first, high initiation, elongation, and termination rates (efficient usage and recycling of ribosomes); and second, low endogenous ribonuclease activity (retention of heavy polysomes for prolonged time).

There is an additional explanation for the dramatic improvement of protein synthesis after washing of the embryos. Thionins are a group of small basic and cysteine-rich proteins, originally purified as antifungal proteins from a variety of plants, including wheat seeds (32). Wheat γ-thionin is known to be in the endosperm of seeds (33), and, recently, Brummer *et al.* have shown in a wheat germ translation system that α- and β-thionin from barley endosperm are potent inhibitors of protein synthesis initiation (34). In addition, several ribonucleases have been reported in the endosperm of the seeds (35). Thus, it is possible that the washing of the embryos resulted in elimination of thionin and ribonucleases as well as tritin.

The Continuous-Flow Cell-Free System on a Preparative Scale. After establishing a procedure for the preparation of highly active wheat embryo extract, we addressed its possible application for the large scale production of protein. For this purpose, we chose a dialysis system because of its continuous supply of substrates and continuous removal of small byproducts (12). With DHFR mRNA as template, protein synthesis worked efficiently, as demonstrated by a Coomassie blue stained gel (Fig. 3*A*, arrow). Densitometric quantitation as well as a direct determination of purified DHFR revealed that the reaction proceeded up to 60 h, yielding 4 mg of enzyme in a 1-ml reaction (Fig. 3*C*). This yield was achieved when the system was supplemented with fresh mRNA every 24 h; without the addition of fresh mRNA, the reaction ceased after 24 h and yielded 1 mg of DHFR (Fig. 3 *B* and *C*, open circles). When aliquots of the reaction mixtures were withdrawn after 48 and 60 h and then were incubated in the presence of a low dose of cycloheximide for an additional 1 h, sucrose gradient centrifugation revealed polysome formation (Fig. 2*C d* and *e*). This is a direct indication of a robust system with high translational activity. The product has a similar specific activity as the authentic enzyme, 15.3 vs. 19.1 units/mg (9).

As shown in Fig. 4, the system also synthesized proteins of higher molecular weight in a preparative scale: 1.1 mg of luciferase (65 kDa), 1.2 mg of GFP (45 kDa). These proteins had the same or even higher specific activity compared with commercially available recombinant forms (Fig. 4 *A* and *B*; see *Materials and Methods*). Furthermore, the 126-kDa replicase of TMV, a major genome product (36) during infection, was produced with a yield of as much as 0.6 mg (Fig. 4). The synthesis proceeded for up to 72 h, as shown by the increase in intensity of the Coomassie brilliant blue-stained bands. This point was confirmed by autoradiography and analysis of amino acid incorporation: [^{14}C]leucine was added after 52 h, samples were withdrawn at 60 and 72 h, and the samples were analyzed by SDS gel electrophoresis and autoradiography (Fig. 4). Densitometric quantitation of the bands showed linear synthesis: The photostimulated luminescence of the sample after 8 h of synthesis (at the 60-h time point) was 186, and after 20 h (at the 72-h point) it was 465, even though the rate of protein synthesis as measured by leucine incorporation was 21% of the rate at the beginning of incubation. This is another direct evidence of the robustness of

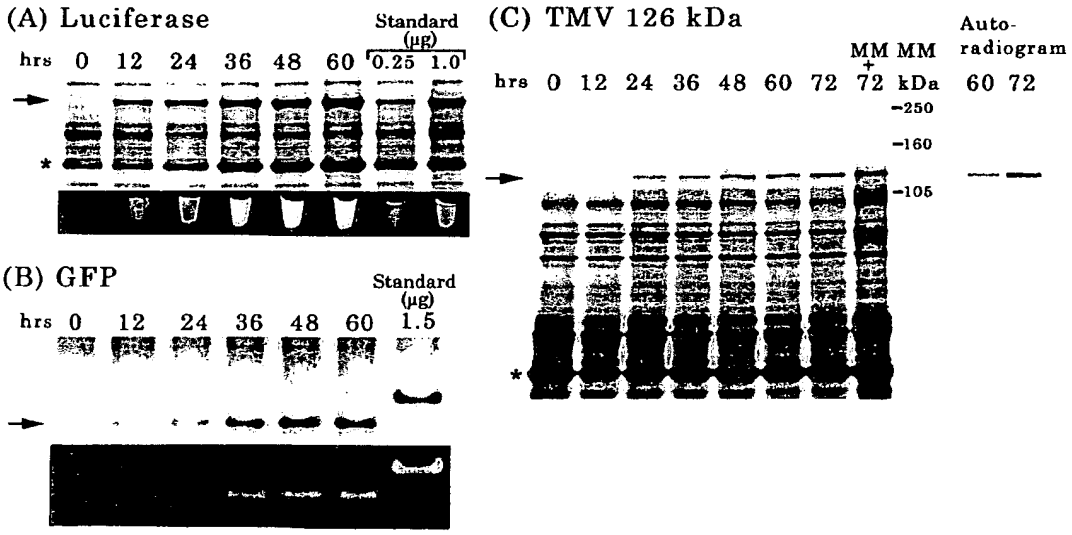


Fig. 4. Synthesis of luciferase (*A*), GFP (*B*), and 126-kDa TMV protein (*C*) in the dialysis system. Samples were analyzed as described in *Materials and Methods*. The standard samples were prepared by mixing a reaction mixture without mRNA with known amounts of luciferase or GFP before loading onto the gel. For the autoradiogram in *C*, [^{14}C]leucine was added at 52 h, and samples were withdrawn after an additional 8 h (60 h total) or 20 h (72 h). Authentic GFP migrates slower than the cell-free product on the native gel, which is attributable to different amino acid compositions because both proteins work as a monomer form. Products and supplemented creatine kinase are marked with arrows and asterisks, respectively.

the system and its efficiency in synthesizing even a 126-kDa protein for 3 days.

The structures of 5′- and 3′-untranslated regions are important for the efficiency of initiation and termination and also for the stability of mRNA (37). The mRNA constructs used here were not optimized in this respect, and we believe that the yields in our experiments do not, therefore, necessarily reflect maximum capacity. Efficient mRNA translation and its regulation requires a series of protein–mRNA and protein–protein interactions (37), and Wells *et al.* have recently shown the circularization of mRNA *in vitro* (38). Our method provides, in addition to its protein synthesis capacity, the opportunity to study translation itself, including the phenomenon of circular mRNA or the characterization of untranslated regions of mRNA in terms of efficient initiation or stability.

We show here that removal of endosperm contaminants, which contain protein synthesis inhibitor(s), from the embryo fraction improves protein synthesis in a wheat germ cell-free system. The improvement likely is caused by increased translational activity resulting from elimination of inhibitors of initiation (e. g. the thionins) and ribonucleases, as well as elimination of the RIP tritin. It is generally believed that cell-free translation systems are inherently unstable, but our results demonstrate the opposite: The translational apparatus appears to be very stable, *in vitro* and presumably also *in vivo*. We believe that our results shed light on the biological function of the nearly ubiquitous plant RIPs. We propose that plants acquired during evolution a suicide system useful to prevent larger damage and that because of its stability the translational machinery is the most important target of a suicide system. Viral attack would be one instance in which this suicide mechanism is employed. Ribosomes are a popular target of antibiotics also, emphasizing their central role in cell metabolism. The observed high stability of the translational apparatus might be an essential requirement for the evolution of life: Certain basic physiological processes such as protein synthesis might be required to function even in adverse conditions.

It is likely that the strategy that we followed to improve the wheat cell-free system, i.e., the inactivation of the translational suicide system, is successful with other systems as well. For instance, the widely used cell-free system from *E. coli* contains high ribonuclease activity and is hampered by a low efficiency in the translation of large mRNAs. Because of significant levels of template degradation, *E. coli* systems are limited when selecting large polypeptides for polysome display.

Our protein synthesis system has several advantages compared with existing systems in addition to its high efficiency: As a eukaryotic system, it is more amenable to the production of eukaryotic proteins from their natural mRNAs: i.e. no cDNA modification is needed; the system can produce high molecular weight proteins; because of little template degradation, it is useful for polysome display (39); and proteins that would normally interfere with cell physiology can be synthesized. Additionally, it should be a useful tool in the study of translation itself.

This work was supported by The Japan Society for the Promotion of Science Grant JSPS-RFTF 96100305 (to Y.E.).

1. Golf, S. A. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 4508–4515.
2. Chrunyk, B. A., Evans, J., Lillquist, J., Young, P. & Wetzel, R. (1993) *J. Biol Chem.* **268,** 18053–18061.
3. Henrich, B., Lubitz, W. & Plapp, R. (1982) *Mol. Gen. Genet.* **185,** 493–497.
4. Kurland, C. G. (1982) *Cell* **28,** 201–202.
5. Pavlov, M. Y. & Ehrenberg, M. (1996) *Arch. Biochem. Biophys.* **328,** 9–16.
6. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2330–2334.
7. Spirin, A. S., Baranov, V. I., Ryabova, L. A., Ovodov, S. Yu. & Alakhov, Yu. B. (1988) *Science* **242,** 1162–1164.
8. Baranov, V. I., Morozov, I. Yu., Ortlepp, S. A. & Spirin, A. S. (1989) *Gene* **84,** 463–466.
9. Endo, Y., Otsuzuki, S., Ito. K. & Miura, K. (1992) *J. Biotechnol.* **25,** 221–230.
10. Kigawa, T. & Yokoyama, S. (1991) *J. Biochem.* **110,** 166–168.
11. Kim, D.-M., Kigawa, T., Choi, C.-Y. & Yokoyama, S. (1996) *Eur. J. Biochem.* **239,** 881–886.
12. Kigawa, T., Yabuki, T., Yoshida, Y., Tsutsui, M., Ito, Y., Shibata, T. & Yokoyama, S. (1999) *FEBS Lett.* **442,** 15–19.
13. Kawarasaki, Y., Kawai, T., Nakano, H. & Yamane, T. (1995) *Anal. Biochem.* **226,** 320–324.
14. Endo, Y., Mitsui, K., Motizuki, M. & Tsurugi, K. (1987) *J. Biol. Chem.* **262,** 5908–5912.
15. Endo, Y. & Tsurugi, K. (1987) *J. Biol. Chem.* **262,** 8128–8130.
16. Wool, I. G., Glück, A. & Endo, Y. (1992) *Trends Biochem. Sci.* **17,** 266–269.
17. Barbieri, L., Battelli, M. G. & Stirpe, F. (1993) *Biochim. Biophys. Acta* **1154,** 237–282.
18. Taylor, S., Massiah, A., Lomonossoff, G., Robert, L. M., Lord, J. M. & Hartely M. R. (1994) *Plant J.* **5,** 827–853.
19. Ready, M. P., Brown, D. T. & Robertus, J. D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5053–5056.
20. Massiah, A. J. & Hartely, M. R. (1995) *Planta* **197,** 633–640.
21. Stewart, T. S., Hruby, D. E., Sharma, O. K. & Robert, W. K. (1977) *Biochim. Bipphys. Acta* **479,** 31–38.
22. Taylor, B. E. & Irvin, J. D. (1990) *FEBS Lett.* **273,** 144–146.
23. Yoshinari, S., Koresawa, S., Yokota, S., Sawamoto, H., Tamura, M. & Endo, Y. (1997) *Biosci. Biotechnol. Biochem.* **61,** 324–331.
24. Hase, M., Endo, Y. & Natori, Y. (1982) *J. Biochem.* **91,** 1457–1465.
25. Chiu, W.-L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. & Sheet, J. (1996) *Curr. Biol.* **6,** 325–330.
26. Hamamoto, H., Sugiyama, K., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y. & Okada, Y. (1999) *Bio/Technology* **11,** 930–932.
27. Erickson, A. H. & Blobel G. (1983) *Methods Enzymol.* **96,** 38–50.
28. Aleen, R. C. (1986) *Methods Enzymol.* **133,** 449–493.
29. Johnston, F. B. & Stern, H. (1957) *Nature (London)* **179,** 160–161.
30. Hirao, I., Yoshinari, S., Yokoyama, S., Endo, Y. & Ellington A. D. (1997) *Nucleic Acids Symp. Ser.* **37,** 283–284.
31. Lodish, H. F., Housman, D. & Jacobsen, M. (1971) *Biochemistry* **10,** 2348–2356.
32. Bohlmann, H. (1994) *Crit. Rev. Plant Sci.* **13,** 1–16.
33. Colilla, F. J., Rocher, A. & Mendez. E. (1990) *FEBS Lett.* **270,** 191–194.
34. Brummer, J., Thole, H. & Kloppstech, K. (1994) *Eur. J. Biochem.* **219,** 425–433.
35. Matsushita, S. (1959) *Memoirs Res. Inst. Food Sci., Kyoto Univ.* **19,** 1–4.
36. Dawson, W. O. (1992) *Virology* **186,** 359–367.
37. Sachs, A. B., Sarnow, P. & Hentze, M. W. (1997) *Cell* **89,** 831–835.
38. Wells, S. E., Hillner, P. E., Vale, R. D. & Sachs, A. B. (1998) *Mol. Cell* **2,** 135–140.
39. Mattheakis, L. C., Bhatt, R. R. & Dower, W. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9022–9026.
40. Yoshinari, S., Koresawa, S., Yokota, S., Sawamoto, H., Tamura, M. & Endo, Y. (1997) *Biosci. Biotechnol. Biochem.* **61,** 324–331.

crocentrifuge tube, and add 0.75 ml of H_2O to reduce salt concentration. Centrifuge at 12,000 *g* for 5 min to remove debris, and transfer supernatant to clean siliconized glass or plastic tubes. Precipitate RNA with 2.5 volumes of ethanol (overnight at −20°). One or two additional ethanol precipitations from 0.2 *M* sodium acetate are required to remove residual NaCl, formamide, and SDS. RNA mass recovery is routinely 70–80%, while recovery of biological activity is higher than 60%.[39]

Saris *et al.*[40] have recently described the *in situ* translation of mRNA bound to ion-exchange paper, which expands the potential versatility of the blotting techniques described here.

[39] P. Lizardi and R. Binder, manuscript in preparation.
[40] C. J. M. Saris, H. J. Franssen, J. H. Heuyerjans, J. van Eenbergen, and H. P. J. Bloemers, *Nucleic Acids Res.* **10**, 4831 (1982).

[3] Cell-Free Translation of Messenger RNA in a Wheat Germ System[1]

By ANN H. ERICKSON and GÜNTER BLOBEL

Cell-free extracts of wheat germ embryos, containing ribosomes and soluble factors, support the *in vitro* translation of a wide variety of mRNAs into protein. The translation apparatus is sufficiently conserved so that a cell-free system can translate both prokaryotic and eukaryotic mRNAs with fidelity. The wheat germ system has been used widely because it is relatively easy to prepare and contains a relatively low amount of endogenous mRNA. The methods described below are modifications of those previously described by Marcus *et al.*[2] and Roberts and Paterson.[3] They have been successfully applied to the *in vitro* synthesis of proteins that each comprise several percent of total cellular protein, such as globin, prolactin, and the caseins, as well as to the synthesis of the lysosomal proteins cathepsin D,[4] β-glucuronidase,[5] and glucocerebrosidase,[5] which each comprise less than 0.1% of total cellular protein.

[1] Supported by Grant No. NP268B from the American Cancer Society.
[2] A. Marcus, D. Efron, and D. P. Weeks, this series, Vol. 30, p. 749.
[3] B. E. Roberts and B. M. Paterson, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2330 (1973).
[4] A. H. Erickson and G. Blobel, *J. Biol. Chem.* **254**, 11771 (1979).
[5] A. H. Erickson and G. Blobel, in preparation.

Source of Wheat Germ

Batches of fresh wheat germ are generally available without charge from large commercial mills, such as General Mills, Vallejo, California or Pillsbury Company, Minneapolis, Minnesota. Since the wheat strains in these batches are variable, it is best to request that several 1-lb samples be taken and sent over a period of time. The variability in the activity of the wheat germ is not well understood, but apparently relates to the wheat strains being processed by the mill. Mills frequently blend the germ from several types of wheat, and the composition of the blend varies with the strains of wheat available at the time. The germ retains activity for over a year when stored in a vacuum desiccator at 4° or in aliquots at −70°.

Flotation of Wheat Germ

Solvent flotation is used to enrich the wheat germ for viable, intact embryos.[6] Reagent-grade cyclohexane (240 ml) and carbon tetrachloride (600 ml) are stirred until no schlieren mixing lines are visible. About 40 g of wheat germ are added, and the mixture is stirred gently with a glass rod. The damaged embryos and contaminating endosperm fragments are allowed to settle away from intact, floating embryos for 2–3 min. The floating germ is collected in a large Büchner or sintered-glass funnel in a fume hood and allowed to dry by pulling air through the funnel for 30 min. About 20–40% of the wheat germ should float. If the recovery is much lower, the solvent ratio can be altered by increasing the amount of carbon tetrachloride added until about 30% of the germ floats. Although the altered solvent ratio allows collection of active germ, the most active preparations are generally obtained when the 1 : 2.5 ratio of cyclohexane to carbon tetrachloride floats approximately 30% of the germ. The organic solvent mixture may be reused for consecutive flotations. Floated germ should be stored in a vacuum desiccator at 4° or in aliquots at −70°.

Preparation of Wheat Germ Extract

The wheat germ extract to be used in the *in vitro* translation system is prepared in three operations: homogenization of the floated germ, centrifugation of the homogenate, and gel filtration of the supernatant. All these procedures should be carried out in the minimum amount of time in a 4° cold room and should employ sterilized buffers and glassware heat-treated at 150° overnight to minimize contamination of the extract by

[6] F. B. Johnston and H. Stern, *Nature* (*London*) **179**, 160 (1957).

RNases. These and all subsequent stock solutions should be made with double-distilled water and should be Millipore-filtered to maximize purity.

Preparation of Buffers

Homogenization buffer contains the following five solutes at the concentrations indicated:

Potassium acetate, 100 m*M*
Magnesium acetate, 1 m*M*
Calcium chloride, 2 m*M*
N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 40 m*M*, pH 7.5
Dithiothreitol (DTT), 4 m*M*

Column buffer contains the following four solutes at the concentrations indicated:

Potassium acetate, 100 m*M*
Magnesium acetate, 5 m*M*
HEPES, 40 m*M*, pH 7.5
Dithiothreitol (DTT), 4 m*M*

Each buffer is prepared from stock solutions without DTT, adjusted to pH 7.6 with KOH, and sterilized in an autoclave. Just before use, DTT powder is added and the buffer is cooled to 4°. Anions should be acetate rather than chloride. At concentrations higher than the cytoplasmic level of 70–80 m*M*, Cl^- severely inhibits the binding of mRNA to ribosomes to form initiation complexes.[7] Use of the acetate anion allows the K^+ concentration in the *in vitro* translation system to be as high as the physiological concentration.

Homogenization of the Wheat Germ

A mortar and a pestle are heat-treated, cooled to room temperature, and chilled with liquid N_2. Floated wheat germ (3 g) is added and ground (usually within 1–2 min) to a fine powder in liquid N_2.[8] The powdered wheat germ is transferred to a second heat-treated mortar resting in an ice bath and ground (again for 1–2 min) in homogenization buffer (10 ml added in three increments) until a thick paste is obtained. Powdering the wheat germ in liquid N_2 presumably minimizes the extent of enzymic degradation occurring between cellular disruption and exposure to buffer.

[7] L. A. Weber, E. D. Hickey, P. A. Maroney, and C. Baglioni, *J. Biol. Chem.* **252**, 4007 (1974).

[8] A. R. Grossman, S. G. Bartlett, G. W. Schmidt, J. B. Mullet, and N.-H. Chua, *J. Biol. Chem.* **257**, 1558 (1982).

Centrifugation of the Homogenate

The homogenate is scraped into a chilled centrifuge tube with a heat-treated spatula and spun at 4° for 10 min at 23,000 *g*. The supernatant is transferred with a heat-treated Pasteur pipette to a clean tube. Care is taken to avoid transferring the floating lipid layer into the tube. The centrifugation step is repeated. The supernatant recovered after the second centrifugation is traditionally known as the S23 wheat germ extract. Its volume is measured.

Gel Filtration of the Supernatant

Gel filtration of the wheat germ extract reduces the concentration of free amino acids, which compete with radiolabeled amino acids for incorporation into protein during *in vitro* translation of mRNA. Isotope dilution assays, which determine the rate of protein synthesis in aliquots of wheat germ extract that contain the same amount of radioactive amino acid but different amounts of added unlabeled amino acid, indicate that gel filtration does not completely deplete free amino acids from the wheat germ extract.[9] The reason for this is unknown. Free amino acids might conceivably be generated by proteolysis during the *in vitro* translation reaction.

A glass column (1.7 × 45 cm) containing a bed of Sephadex G-25 fine (1.7 × 33.3 cm) is equilibrated with sterile column buffer. The wheat germ supernatant is loaded onto the column, and the column is eluted with column buffer under gravity flow. The brownish solution eluting just behind the void volume is collected in approximately 15 fractions (each 2 ml). The slower-running yellow pigment is discarded. The most opaque fractions are pooled to provide a solution whose volume is approximately equal to the volume loaded onto the column. The final yield from 3 g of wheat germ should be about 7 ml. This solution is centrifuged at 4° for 10 min at 23,000 *g*. The scale of the whole procedure can be increased, using more wheat germ and a larger G-25 column. Autoclaved microfuge tubes containing 100–200-μl aliquots of gel-filtered wheat germ extract are immediately frozen in liquid N_2. The wheat germ extract is stable for at least a year when stored at −70°.

Components of the Translation Assay

Amino Acids and Energy-Generating System

Stock solutions are prepared at the following concentrations:
Adenosine 5'-triphosphate (ATP), 0.1 *M* (disodium salt)

[9] P. Walter, I. Ibrahimi, and G. Blobel, *J. Cell Biol.* **91**, 545 (1981).

Guanosine 5'-triphosphate (GTP), 0.02 *M* (sodium salt)
Creatine phosphate (CP), 0.6 *M* (disodium salt hydrate)
[^{35}S]Methionine or a desired tritiated amino acid
The 19 other amino acids, 1 m*M* each
Potassium hydroxide, 1.0 *M*
Creatine phosphokinase (CPK) from rabbit muscle, 8 mg/ml

The ATP, GTP, CP, and CPK stock solutions should be stored in small aliquots at −20°. All except the CPK solution can be refrozen. The stock solution of 19 amino acids is conveniently prepared from 20 m*M* stock solutions of each amino acid. All amino acids are soluble at 20 m*M* in distilled water except Phe, Asn, Ile, Trp, Val, Glu, and Asp, which require 0.01 *N* HCl, and Tyr, which is soluble in 0.1 *N* HCl. The stock solution of the 19 combined amino acids and the individual 20 m*M* amino acid solutions should be stored at −20° and may be refrozen. Translation-grade [^{35}S]methionine (1000 Ci/mmol, 1 mCi/110 μl) should be stored in small aliquots at −70° to minimize oxidation, which may occur on repeated freezing and thawing and on exposure to air. If the protein under study lacks methionine, [^{35}S]cysteine, available at a higher specific activity (500 Ci/mmol) than tritiated amino acids, may be useful. If the protein is unusually rich in a particular amino acid, even one available only at a low specific activity, this may be a useful choice for incorporation, as the background of labeling of total cellular protein should be low relative to incorporation into the particular protein under study.

A 50-μl mixture of the above components, which is enough for twelve 20-μl translation reactions, is prepared by mixing, in the order given, 3 μl of 0.1 *M* ATP, 1 μl of 0.02 *M* GTP, 4 μl of 0.6 *M* CP, 5 μl of the 19 amino acid solution, 9 μl of water, 25 μl of [^{35}S]methionine, and 1 μl of 1.0 *M* KOH. The pH of this mixture is checked by spotting 1 μl on pH paper. If the pH is less than 7.0, the solution is adjusted with additional KOH to pH 7–7.6. Finally, 2 μl of the CPK stock solution are added. This resulting solution can be stored at −70° and reused by addition of 2 μl of fresh CPK stock solution, but this generally results in a 10–20% decrease in amino acid incorporation. In the final translation reaction, the mixture is diluted 1 : 5, giving final concentrations of 1.2 m*M* ATP, 0.08 m*M* GTP, 9.6 m*M* CP, 20 μ*M* of each of the 19 amino acids, CPK at 64 μg/ml, and [^{35}S]methionine at 910 μCi/ml.

The amount of radioactivity incorporated into protein can be increased by substituting the radiolabeled amino acid solution for the portion of water. In addition, a desired quantity of the radiolabeled amino acid may be lyophilized in a microfuge tube and resuspended in 25 μl of distilled water. The other components of the mixture may be added directly to this tube. Concentration by lyophilization is especially important

to maximize the incorporation of an amino acid that is available only at a dilute concentration. If the radioactive amino acid is supplied in a salt solution, the salt concentration should be taken into account when preparing the compensating buffer (see below). A methionine concentration of 2–3 mCi per milliliter of total translation mix is quite adequate for the *in vitro* synthesis of lysosomal enzymes,[4] which each comprise less than 0.1% of total cellular protein.

Wheat Germ Compensating Buffer

The K^+ and Mg^{2+} concentrations of the wheat germ translation system have dramatic effects on the efficiency of translation of particular mRNAs.[10,11] The S23 wheat germ extract prepared as described above contains 100 m*M* K^+ and 5 m*M* Mg^{2+}. The compensating buffer is used to adjust the ion concentrations of the total translation reaction to an optimum that must be determined for each mRNA being translated. The optimal concentrations are generally 130–140 m*M* for K^+ and 2.0–2.5 m*M* for Mg^{2+}. It is important to examine, by polyacrylamide gel electrophoresis, the effect of ion concentrations on the radiolabeled amino acid incorporated into the protein under study rather than be guided by the amount of radiolabeled amino acid incorporated into total protein.

Compensating buffer, which is diluted 1 : 10 in the final translation mixture, contains the following four solutes at the concentrations indicated:

Potassium acetate, 1.0 *M*
Magnesium acetate, 5 m*M*
Spermine, 0.8 m*M*
Dithiothreitol (DTT), 20 m*M*

This compensating buffer is used when the final translation mixture contains 40% of the S23 wheat germ extract. When the final translation mixture contains 50% wheat germ extract, the magnesium acetate is omitted from the compensating buffer and the potassium acetate concentration is decreased to 0.9 *M*. In both cases, the concentration in the final translation mixture will be 140 m*M* for K^+ and 2.5 m*M* for Mg^{2+}. Spermidine, at a concentration approximately 10 times that used for spermine,[11] can replace spermine in the compensating buffer and may improve translation of certain mRNAs.[12] The compensating buffer can be stored in aliquots at −70°.

[10] P. T. Lomedico and G. F. Sunders, *Science* **198**, 620 (1977).
[11] T. P. H. Tse and J. M. Taylor, *J. Biol. Chem.* **252**, 1272 (1977).
[12] K. E. Mostov and G. Blobel, this series, Vol. 98 [40].

mRNA

Total RNA or poly(A)-containing mRNA, prepared as described in this volume,[13] can be translated in a wheat germ system. When the mRNA under study is present at low concentration in a given tissue, it is advantageous to enrich its concentration by translating a particular size class of RNA, which is isolated from a sucrose gradient or a sizing gel.

The RNA is suspended in sterile distilled water at a concentration of approximately 1 mg/ml (20 A_{260}/ml). The optimal final RNA concentration in the translation reaction should be determined for each RNA preparation to be translated, but is generally 50–150 μg/ml (1–3 μl of RNA at 20 A_{260}/ml per 20 μl of total translation mixture). Higher RNA concentrations often result in decreased protein synthesis, probably due to inhibitors in the RNA preparation. Dilute RNA preparations may be concentrated by ethanol precipitation or lyophilization.

Wheat Germ Extract

The optimal concentration of S23 wheat germ extract should be determined for each RNA to be translated, but is generally either 40 or 50% of the total translation mix. Appropriate concentrations of K^+ and Mg^{2+} in the compensating buffer are used to adjust for the percentage of the wheat germ extract present.

The low concentration of endogenous mRNA present in the wheat germ extract competes with the added RNA for ribosomes and factors required for translation. It is advantageous to reduce the concentration of endogenous RNA by treating with nuclease.[14] Micrococcal nuclease from *Staphylococcus aureus* is dissolved in distilled water at a concentration of 1875 units/ml. Aliquots of the diluted enzyme are stored at −70° and can be refrozen. This nuclease solution (2 μl) is mixed with 100 μl of freshly thawed wheat germ extract and 2 μl of 0.1 *M* $CaCl_2$ and incubated for 5 min at 21° with occasional mixing. The reaction is terminated by transfer of the tube to an ice bath and addition of 4 μl of 0.1 *M* EGTA, which chelates the Ca^{2+} ion required for nuclease activity. Nuclease-treated wheat germ loses only 5–10% of its activity when refrozen immediately in liquid N_2. Alternatively, wheat germ may be nuclease-treated in large batches immediately after elution from the Sephadex G-25 column and prior to centrifugation and freezing in aliquots.

[13] P. M. Lizardi, this volume [2].
[14] H. R. B. Pelham and R. J. Jackson, *Eur. J. Biochem.* 67, 247 (1976).

Optional Components

Transfer RNA. For some batches of wheat germ, the efficiency of translation of certain messenger RNAs can be increased by adding a mixture of heterologous transfer RNAs, such as commercial calf liver tRNA. The optimal concentration of added tRNA is determined by titration. It usually lies between 50 and 500 μg of tRNA per milliliter of total translation mixture. Use of higher concentrations can decrease the net incorporation of radiolabeled amino acids into protein. The effect on the net incorporation into the protein under study should be the guiding factor in choosing the optimal tRNA concentration. Depending on the amount of tRNA added, it may be necessary to alter the final Mg^{2+} concentration in the translation.

Ribonuclease Inhibitor. The addition of human placental RNase inhibitor to the wheat germ system can increase the yield of high molecular weight proteins.[15] The magnitude of this increase varies with different batches of wheat germ. This RNase inhibitor can be prepared by affinity chromatography on RNase A–Sepharose.[16] The optimal concentration of RNase inhibitor for a given translation system is determined by titration. It usually lies between 2 and 10 μg of RNase inhibitor per milliliter of total translation mixture.

Protease Inhibitors. Selected protease inhibitors that do not inhibit protein synthesis may be added at the following final concentrations: pepstatin A, 0.1 μg/ml; chymostatin, 0.1 μg/ml; antipain, 0.1 μg/ml; leupeptin, 0.1 μg/ml; and Trasylol, 10 units/ml.[9]

Microsomal Membranes. Microsomal membranes from the endoplasmic reticulum can translocate nascent secretory and lysosomal proteins, which usually results in cleavage of the amino-terminal signal sequence and addition of high-mannose carbohydrate chains, and can integrate newly synthesized membrane proteins. Preparation of these membranes and their use in the wheat germ system are described in this volume.[17]

Scale of the Reaction

The scale of the translation reaction required for detection of the protein under study depends upon the amount of its mRNA in the total RNA preparation. A major secretory protein, which usually comprises several percent of the total cellular protein, can normally be visualized by

[15] G. Scheele and P. Blackburn, *Proc. Natl. Acad. Sci. U.S.A.* 76, 4898 (1979).
[16] P. Blackburn, *J. Biol. Chem.* 254, 12484 (1979).
[17] P. Walter and G. Blobel, this volume [6].

polyacrylamide gel electrophoresis of a 20-μl reaction mixture containing [^{35}S]methionine, followed by autoradiography for 24 hr. In contrast, a lysosomal protein comprising less than 0.1% of the total cellular protein may require immunoprecipitation from a 100-μl reaction mixture and fluorography for 2–3 weeks.

Order of Component Addition

The components of the translation mixture are generally combined in a tube chilled in an ice bath in the following order: 4 μl of mixture of radiolabeled amino acid, 19 unlabeled amino acids, ATP, GTP, CP, and CPK; 2 μl of compensating buffer; distilled water; transfer RNA (if present); RNase inhibitor (if present); 8 μl of nuclease-treated S23 wheat germ extract; 1–3 μl of mRNA solution; microsomal membranes (if present).

The volume of distilled water added is chosen to bring the total volume of the translation mixture to 20 μl. Plastic microfuge tubes with conical bottoms are convenient reaction vessels. After addition of each reagent, the mixture is mixed thoroughly but gently. Brief centrifugation in a microfuge eliminates bubbles and ensures that no liquid remains as beads on the walls of the vessel. In order to maximize reproducibility when the amount of one reagent is being varied, the other reagents are combined, mixed, and divided equally among a series of tubes containing varying amounts of the last reagent.

Time and Temperature of the Reaction

At the beginning of the reaction period, a portion (2–5 μl) of the translation mixture is spotted on a filter disk to measure the background radioactivity (see below). The translation mixture is generally incubated at 25–27° for 60–90 min. Longer reaction periods often result in decreased net incorporation of radiolabeled amino acids into protein, presumably owing to degradation of newly synthesized polypeptide chains. At higher temperatures, protein synthesis proceeds faster but terminates sooner, which also decreases the net incorporation.

Termination of the Reaction

Cooling the reaction mixture in an ice bath is sufficient to terminate the translation reaction. Permanent termination is achieved by detergent denaturation or trichloroacetic acid precipitation of the protein in the reaction mixture.

Analysis of Translation Products

Incorporation of Radiolabel into Total Protein

The extent of incorporation of the radiolabeled amino acid into total protein can be determined by measuring the amount of radioactivity incorporated into acid-precipitable protein, using a modification of the method of Mans and Novelli.[18] This assay provides an overall estimate of the efficiency of the RNA translation. A small aliquot (2–5 μl) of the translation mix is spotted on a Whatman 3 M filter disk (2.5 cm in diameter) and dropped into a beaker of 10% trichloroacetic acid resting in an ice bath. Several filter disks can be treated in the same beaker, using 3 ml of trichloroacetic acid per filter. Handling is facilitated by insertion of a straight steel pin into the paper disk, which can be labeled with pencil. After 30 min for precipitation of protein, the 10% trichloroacetic acid is decanted and 5% trichloroacetic acid is added and heated to boiling on a hot plate in a fume hood for 15 min. If the filters are boiled too long, the concentration of the acid increases and protein hydrolysis can occur, which decreases the radioactivity in the precipitated protein. The hot 5% trichloroacetic acid is decanted, and the filters are rinsed three times in fresh 5% acid. The filters are immersed in a 1 : 1 solution of ethanol and ether for about 5 min. The solvent mixture is then decanted and ether is added. After 5 min, the ether is decanted and the filters are air-dried in a fume hood and counted in a toluene-based scintillation fluid. After counting and removal of the filter disks, the scintillation vial and fluid can be reused numerous times without significantly increasing the background radioactivity.

Definitions and Controls

Several control experiments are normally carried out to facilitate the interpretation of the translation results.

1. Background radioactivity is the percentage of trichloroacetic acid precipitable radioactivity at the beginning of the translation reaction. This control measures the binding of the free radiolabeled amino acid to protein in the wheat germ extract or to the filter disk.
2. Stimulation of the incorporation of radiolabeled amino acid into newly synthesized protein is defined as the ratio of the incorporation due to a specific source of mRNA divided by the background radioactivity.
3. Endogenous stimulation is the ratio of radioactivity incorporated

[18] R. J. Mans and G. D. Novelli, *Arch. Biochem. Biophys.* 94, 48 (1961).

owing to the presence of endogenous mRNA in the wheat germ extract divided by the background radioactivity. This control is measured by omitting the mRNA under study from the translation mixture.

4. Efficiency of nuclease treatment is the percentage decrease in endogenous stimulation due to treatment of the S23 wheat germ extract with nuclease. It is measured by determining the incorporation using both nuclease-treated and untreated wheat germ extract.
5. Standard net stimulation is the net incorporation due to the presence of a standard mRNA minus the endogenous stimulation. This control is especially helpful when changing any components of the reaction. It is measured by subtracting the endogenous stimulation from the observed stimulation. It is useful to set aside aliquots of a particular mRNA preparation for use as a standard mRNA.
6. If a new RNA preparation proves to be inactive, it is helpful to translate a mixture of the new mRNA and the standard mRNA. If the observed stimulation is less than the standard stimulation, the new RNA preparation probably contains inhibitors of protein synthesis. Further purification of the RNAs should be helpful in this case.

Typical mRNA-Specific Stimulation of Protein Synthesis

The stimulation of protein synthesis varies greatly with the mRNA being translated. The average standard net stimulation is 40- to 100-fold but can be as high as 400-fold using certain RNAs under optimal conditions.

So many factors affect amino acid incorporation in the wheat germ system that it is difficult to generalize. Under our conditions, the background radioactivity using [^{35}S]methionine is about 5000 cpm per 2.5 μl of translation mixture. Endogenous stimulation using nuclease-treated wheat germ extract is normally 4- to 5-fold, or 20,000–25,000 cpm per 2.5 μl. Standard net stimulation by an exogenous mRNA preparation will be 40- to 100-fold, or 200,000–500,000 cpm per 2.5 μl. Highly efficient standard net stimulation can be 400-fold, or 2,000,000 cpm per 2.5 μl.

The secretory protein prolactin comprises several percent of the total protein of bovine pituitary glands. Total RNA from this tissue produces about a 40-fold standard net stimulation in radioactive amino acid incorporation in the wheat germ system. It has been estimated[19] that approximately 10 fmol of preprolactin are synthesized in a 25-μl wheat germ *in vitro* translation assay using bovine pituitary RNA.

[19] P. Walter and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7112 (1980).

Polyacrylamide Gel Electrophoresis (PAGE) of the Translation Products in Sodium Dodecyl Sulfate (SDS)

The method most commonly used for examining the proteins produced by cell-free translation is SDS–PAGE. Various aspects of this technique are discussed elsewhere in this volume.[20,21] The products of *in vitro* synthesis are usually analyzed by autoradiography or fluorography because these sensitive techniques for detection of radiolabeled protein are usually necessary to visualize the small amounts of protein synthesized.

If the protein under study is coded for by an RNA comprising a large percentage of the total RNA population of the tissue, such as a secretory protein, it may be possible to detect that protein directly among the total translation products. It is important to run a sample of the proteins produced by endogenous mRNA on the same gel in order to distinguish which protein bands are due to endogenous mRNA in the wheat germ extract. Electrophoresing radiolabeled molecular weight standards allows determination of the molecular weights of particular protein products.

A small aliquot (5 μl) of translation mixture can be diluted with a loading buffer (25 μl) containing 3% SDS and loaded directly on the gel. Dithiothreitol may also be added at a concentration of 20 m*M*. Subsequent alkylation using iodoacetamide (100 m*M*, 30 min, 37°) is often required to prevent oxidation of the thiol groups and re-formation of disulfide bonds.

Large aliquots of translation mixture (>25 μl) may be precipitated with trichloroacetic acid and the pellets resuspended in the SDS loading buffer. Trichloroacetic acid precipitation also reduces the concentration of salts, which may distort the pattern of protein bands in the gels. Since the wheat germ extract contains nonradioactive proteins that are precipitated by trichloroacetic acid and loaded onto the gel along with the radiolabeled translation products, it is important not to precipitate too large a sample of the translation mixture. Loading large amounts of protein in a single gel slot produces distortion of the shape of the protein bands and aberrant electrophoretic migration, and thus incorrect estimates of the molecular weights of certain proteins.

Immunoprecipitation of Specific Translation Products

If the protein under study comprises a small percentage of the total protein of a particular tissue (<1–2%), it is necessary to immunoprecipi-

[20] R. W. Rubin and C. L. Leonardi, this volume [12].
[21] W. M. Bonner, this volume [15].

tate the protein prior to PAGE. Immunoprecipitation of *in vitro* translation products is discussed in this volume.[22]

Other Cell-Free Translation Systems

In principle it should be possible to prepare a cell-free extract for RNA translation from any cell type. In practice, relatively few cell-free systems have been developed for *in vitro* translation. In general, these systems are derived from cells engaged in a high rate of protein synthesis. They include (*a*) rabbit reticulocyte lysate[23]; (*b*) bacterial cell lysate[24]; (*c*) yeast cell lysate[25-27]; (*d*) HeLa, Chinese hamster ovary, or L-cell lysate[28,29]; (*e*) mouse Krebs ascites cell lysate.[30,31]

[22] D. J. Anderson and G. Blobel, this volume [8].
[23] R. J. Jackson and T. Hunt, this volume [4].
[24] P. Green and M. Inouye, this volume [5].
[25] E. Gasior, F. Herrera, I. Sadnik, C. S. McLaughlin, and K. Moldave, *J. Biol. Chem.* **254**, 3965 (1979).
[26] M. F. Tuite, J. Plesset, K. Moldave, and C. S. McLaughlin, *J. Biol. Chem.* **255**, 8761 (1980).
[27] B. Wolska-Mitaszko, T. Jakubowicz, T. Kucharzewska, and E. Gasior, *Anal. Biochem.* **116**, 241 (1981).
[28] M. J. McDowell, W. K. Joklik, L. Villa-Komaroff, and H. F. Lodish, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2649 (1972).
[29] L. A. Weber, E. M. Feman, and G. Baglioni, *Biochemistry* **14**, 5314 (1975).
[30] I. M. Kerr, N. Cohen, and T. S. Work, *Biochem. J.* **98**, 826 (1966).
[31] M. B. Mathews and A. Korner, *Eur. J. Biochem.* **17**, 328 (1970).

[4] Preparation and Use of Nuclease-Treated Rabbit Reticulocyte Lysates for the Translation of Eukaryotic Messenger RNA

By RICHARD J. JACKSON and TIM HUNT

In 1968 two research groups independently found that when rabbit reticulocyte lysates were supplemented with hemin they synthesized protein at the same rate as the intact cell for periods of about 60 min.[1,2] The remarkably high activity of this cell-free system has still not been equaled

[1] S. D. Adamson, E. Herbert, and W. Godschaux, *Arch. Biochem. Biophys.* **125**, 671 (1968).
[2] W. V. Zucker and H. M. Schulman, *Proc. Natl. Acad. Sci. U.S.A.* **59**, 582 (1968).

by extracts of other eukaryotic cells. Whereas these early experiments were concerned only with the translation of endogenous reticulocyte mRNA, it was subsequently shown that exogenous heterologous mRNA is also efficiently and faithfully translated in the lysate.[3] The disadvantage of such experiments is that the added mRNA is translated in competition with the endogenous reticulocyte mRNA, which makes quantitation of the template activity of the exogenous RNA difficult and tedious. The ideal translation system of which we used to dream would be a reticulocyte lysate from which the endogenous mRNA had been removed by fractionation or selective destruction without impairing the intrinsic high activity of the translation machinery. This proved to be easy in practice by using micrococcal endonuclease (EC 3.1.31.1) to destroy the endogenous mRNA.[4] This nuclease has an absolute requirement for Ca^{2+} as cofactor, so that globin mRNA can be destroyed by preincubating the lysate with nuclease and Ca^{2+}. EGTA is then added to chelate the Ca^{2+} and thereby inactivate the nuclease. Such is the specificity of EGTA that it is possible to lower the calcium levels below the level required for enzyme activity without significantly affecting the level of Mg^{2+}, which is critical for high-level protein synthesis. (After addition of EGTA, the concentration of free Ca^{2+} is about 10^{-7} *M*, whereas the Mg^{2+} concentration is reduced by less than 10%.) The resulting nuclease-treated lysate has a very low activity unless eukaryotic mRNAs are added. These are translated with remarkable efficiency, more or less whatever their origin (from yeast to higher plants or mammals). The efficiency of the system is indicated by the recovery of up to 70% of the original activity of the "parent" lysate when globin mRNA is added.[4] The failure to regain 100% activity may stem from the fact that after the nuclease treatment many ribosomes are blocked or stranded on short fragments of globin mRNA. One would not expect these ribosomes to be available for translating added mRNA, though preliminary experiments suggest that they do at least release their incomplete nascent chains during the incubation. We do not know whether they are also released from their mRNA fragments.

In spite of the fact that reticulocytes are highly specialized cells, the translation machinery shows little specialization or preference with regard to initiation of protein synthesis on different eukaryotic mRNAs. It is only with respect to the complement of tRNA species that the reticulocyte lysate shows special properties that may impair its ability to translate heterologous mRNA. The relative abundance of different types of tRNA in reticulocytes has been shown to be highly adapted to the synthesis of

[3] R. D. Palmiter, *J. Biol. Chem.* **248**, 2095 (1973).
[4] H. R. B. Pelham and R. J. Jackson, *Eur. J. Biochem.* **67**, 247 (1976).